DIAGNOSIS OF PREDISPOSITION TO EPILEPSY AND MONITORING OF ANTIEPILEPTIC TREATMENT

FIELD AND BACKGROUND

The present invention is of methods and diagnostic assays for a biochemical approach to the detection of a predisposition to epilepsy and to the clarification of the diagnosis of epilepsy, and for improved monitoring of the treatment regimen for antiepileptic drugs.

Epilepsy is one of the most common chronic neurological disorders. The disease is characterized by recurrent seizures, which originate from abnormal and excessive activity of cerebral neurons and result in a paroxysmal disorganization of brain function.

The different types of epilepsy include partial (symptomatic) and generalized idiopathic seizures. Partial epilepsy is "localization related'' and originates in a limited area of the brain. On the other hand, a specific brain lesion or disease, other than a possible genetic propensity to generate seizures, does not cause the generalized form of epilepsy. Generalized, or grand mal, seizures include tonic-clonic seizures, in which the entire body undergoes convulsions. Left untreated, epilepsy can degenerate into status epilepticus, a potentially fatal neurological emergency [Antiepileptic Drugs; eds. R.H. Levy, R.H. Mattson and B.S. Meldrum; 4th Edition, Raven Press, NY, NY; J. Aicardi, Epilepsy in children, 2d Edition, Press 1994].

Idiopathic epilepsy appears to be a heritable disorder though little is known about the precise genetic or biochemical defects involved (Andermann, 1982; Delgado - Escueta et al, 1986, Anderson, 1982; Anderson et al., 1986; see attached Appendix for complete references). Recent research has indicated the possibility of genetic predisposition to the development of localization-related epilepsy, in particular, post-traumatic epilepsy. In this type of epilepsy, a head injury is the resolving exogenous factor inducing the disease with a low penetration of the pathological hereditary factor.

Over 53 million people world-wide suffer from epilepsy, with 2.5 million who have had, or who will have seizures at some point in the United States of America alone. Epilepsy primarily affects children and young adults. Almost 50% of new epilepsy cases occur prior to age 25. A large number of children and adults have undetected or untreated epilepsy.

Early diagnosis of epilepsy is crucial, since repeated seizures may cause severe brain damage and intellectual deterioration. Learning difficulties, behavior disturbances and poor

social adjustment are associated with epileptic syndromes.

Unfortunately, no predictive in vitro diagnostic test is currently available. At present, diagnosis of epilepsy is based primarily on clinical history, EEG paroxysmal activity and findings on neuroimaging when available. A sensitive, reliable, and non-invasive method for pre-symptomatic diagnosis of epilepsy would undoubtedly benefit individuals in certain groups at high risk of developing epilepsy. Such groups include first and second degree relatives of epileptic patients, babies born prematurely or with a traumatic delivery and febrile convulsive children. Others at risk include those patients who have endured severe cerebral injury or intracranial infections, pregnant women, soldiers or pilots who will be subjected to stressful situations, among others. If available, in vitro pre-symptomatic diagnosis would provide an opportunity in the future for prophylactic treatment for members of these groups at high risk of developing epilepsy.

Antiepileptic drugs (AED) are used to reduce the number and the severity of seizures in patients with epilepsy. Serious side effects are associated with the treatment of epilepsy by currently used AED, with adverse reactions occurring in up to 50% of epileptic patients. Of those patients who are free of seizures because of drug therapy, 31% have complained of memory loss, difficulty in thinking clearly, drowsiness, behavioral changes, clumsiness, lethargy and weight gain. Many patients suffer from epilepsy which is refractory to antiepileptic drugs. Refractory epilepsy accounts for about 30% of non-compliant subjects, of whom 15% cannot achieve full seizure control and 15% do not receive any benefit at all from AED treatment. Monitoring of AED therapy is aimed at the prevention of the conversion of epilepsy into one of the intractable forms of epilepsy.

Currently available methods for monitoring of AED treatment are not sufficiently sensitive or accurate, as they only rely upon the measurement of the concentrations of AED in the blood. The most complicated problems in the effective monitoring of AED treatment include specific responses of the individual to AED intervention; discontinuation of AED therapy in patients, who had been seizure free with AED therapy; and the summed therapeutic and toxic effect of combination of AED. More quantitative measurements are required, which enable AED therapy to be adjusted for the individual, such that the best possible balance between therapeutic efficacy and undesirable side effects is obtained.

Recently, neuroactive properties of the metabolites of the kynurenine pathway of tryptophan degradation - L-KYN, KA, AA, 3HOKYN, 3HOAA, QUIN (Figure 1) - have

attracted considerable attention as endogenous neuroprotective and neurotoxic agents in a number of neurological diseases, including epilepsy. The key metabolites of the kynurenine pathway were shown to be compounds with functionally opposite properties.

Kynurenic acid (KA), as an excitatory amino acid receptor antagonist with antineurotoxic and anticonvulsant activity, effectively blocks the action of NMDA and some other excitatory amino acids in the rat neocortex, hippocampus, n. caudate and spinal cord (Perkins and Stone, 1982; Stone, 1993). In slices of neocortex resected from infants and children with intractable epilepsy, KA applied in the perfusion medium decreased spontaneous burst frequency and reduced burst duration, or even blocked spontaneous bursts of seizure activity completely (Wuarin et al., 1990). The concentration of KA in the blood and extracellular compartment is regulated by the availability of L-KYN. L-KYN effectively crosses the blood-brain barrier (Fukui et al., 1991), and produces a linear dose-dependent increase in KA concentration in the brain (Stone 1993). A relatively low concentration of L-KYN and KA in the CSF (cerebro-spinal fluid) was found both in patients with infantile spasm (Yamamoto, 1991; Yamamoto et al., 1995) and intractable complex partial seizures (Young et al., 1983; Heyes et al.1994).

By contrast to KA, quinolinic acid (QUIN), an excitotoxic agonist of NMDA (N-methyl-D-aspartate) receptor, is the most potent endogenous convulsant discovered so far (Stone, 1993). Upon microionophoretic application, QUIN excites cortical neurons (Stone a. Perkins, 1981; Perkins and Stone, 1983). In appropriate molar ratios, KA completely antagonizes the neurotoxicity of QUIN (Jhamandos et al, 1990; Miranda et al., 1997).

QUIN is synthesized by the enzyme 3HAO (3-hydroxyanthranilic acid oxygenase (EC 1.13.11.6)) from its precursor 3HOAA, and is catabolized by quinolinic acid phosphoribosyltransferase (QPRT; EC 3.2.2.5) within the brain. The activity of these enzymes controls the level of extracellular QUIN. QUIN is thought to be unable to penetrate the blood-brain barrier. Once extracellular levels exceed a critical limit, QUIN may enter brain cells via passive diffusion (Schwarcz et al., 1991).

3-hydroxykynurenine (3-HOKYN) has also been reported to be a convulsant (Lapin, 1981; Guilarte et al.1987; Stone, 1993). When administered intraperitoneally, 3-HOKYN is by far the most potent metabolite for elevating the level of QUIN in the brain (Reinhard et al., 1994). High levels of both 3HOKYN and QUIN were found in the CSF of patients with infantile spasm, the most severe form of epilepsy.

3-hydroxyanthranilic acid (3HOAA), which is formed by kynureninase from 3-HOKYN, is an immediate and efficient precursor of QUIN, which mimics the neurotoxic effect of QUIN. 3HOAA also does not cross the blood-brain barrier. It was shown that in situations where increased quantities of 3-HOAA are available to the cell, a greater amount of QUIN is accumulated by the brain (Gal and Sherman, 1980; Moroni, 1984; Kohler et al., 1989). Though the potency of 3-HOAA is lower than that of QUIN, its maximal neurotoxic effect is comparable in intensity to the neurotoxic effect produced by QUIN (Jhamandas et al., 1990). Unlike 3-HOAA, anthranilic acid (AA) is a neuroprotective compound (Jhamandas, 1990, 1992).

The kynurenine pathway of tryptophan metabolism, as shown in Figure 1, is known to be pyridoxal-phosphate (PLP, vitamin B6)-dependent. Kynureninase (EC 3.7.1.3), the enzyme which catalyzes the synthesis of both AA from L-KYN and 3HOAA from 3HOKYN, is especially sensitive to the supply of PLP (Bender, 1989). Another PLP-dependent enzyme of the kynurenine pathway (with much higher K_M) is kynurenine amino-transferase (KAT; EC 2.6.1.7), which transforms L-KYN to KA, and 3HOKYN to xanthurenic acid as well. Thus, there are at least two points along the kynurenine pathway of tryptophan degradation which are sensitive to the disorders in the metabolism of vitamin B6.

Without wishing to be bound by a single mechanism, according to the hypothesis of the inventors, an inborn error in vitamin B6 metabolism may form the genetic basis of an enhanced seizure sensitivity (Dolina, Kozak 1987; Dolina, 1988, 1992; Dolina et al., 1989, 1993). Therefore, as described with regard to the present invention, the alterations in concentrations of the metabolites of PLP-dependent kynurenine pathway can be used as the biochemical markers of the genetic predisposition to epilepsy. As the concentrations of these metabolites change during AED treatment, these markers in combination with the measurement of the concentration of AED (by any of variety of techniques used for measuring AED) also provides effective monitoring of AED therapy according to the present invention. However, such a use for the detection of the altered concentrations of metabolites has never been taught or suggested by the prior art.

SUMMARY OF THE INVENTION

It is one object of the present invention to provide a biochemical approach for the detection of a genetic predisposition to epilepsy and for the clarification of the diagnosis of

clinical epilepsy itself, for example to confirm the diagnosis of clinical epilepsy already made by another approach or to discriminate between epilepsy and other kinds of convulsions (for example, hysterical convulsions). In particular, the present invention features the detection of the altered biochemical profile of tryptophan metabolites of the kynurenine pathway, which is associated both with clinical epilepsy and with the genetic predisposition to epilepsy.

It is another object of the present invention to provide improved monitoring of epilepsy treatment by AED (antiepileptic drugs).

It is still another object of the present invention to provide a diagnostic system implementing a biochemical approach to pre-symptomatic detection of a genetic predisposition to epilepsy, to the clarification of the diagnosis of clinical epilepsy and to monitoring of epilepsy treatment by AED.

All of these objects of the present invention involve the quantitative assessment of the biochemical condition of the subject by measuring the amount of at least two kynurenine metabolites.

These and other objects of the present invention will be further detailed in the following description, Tables and claims.

Without wishing to be limited by a single mechanism, it should be noted that disturbances in the kynurenine pathway of tryptophan metabolism are found in individuals with clinical epilepsy and with a genetic predisposition to epilepsy. These metabolic disturbances result in alterations to the concentrations of individual kynurenine metabolites, as well as to the ratio of certain metabolites. Certain of these metabolites are known to be neuroprotective, while others are known to be neurotoxic. In particular, the important balance between the level of neuroprotective and neurotoxic metabolites is upset in individuals with seizure disorders. Individuals with epilepsy or with a predisposition to epilepsy have a low ratio of neuroprotective to neurotoxic metabolites, compared to normal individuals. However, if excessive amounts of AED are administered for antiepileptic therapy, then an excessively high ratio of neuroprotective to neurotoxic metabolites results, which is indicative of enzymatic suppression and disruption of the enzymatic balance in the kynurenine metabolic pathway. Thus, these metabolites should preferably be present in a balanced ratio which is indicative of healthy neuronal functioning, and if administered, effective AED therapy.

The alterations in the concentrations of particular metabolites, and especially in the ratio of two or more such metabolites, can be used as biochemical markers for the detection of a predisposition to epilepsy, the clarification of the diagnosis of clinical epilepsy, and for monitoring treatment with antiepileptic drugs.

According to the present invention, there is provided a method for diagnosing a predisposition to epilepsy, or epilepsy itself, in a subject, the method comprising the steps of: (a) obtaining a sample from the subject; (b) measuring a concentration of at least two kynurenine metabolites in the sample; and (c) comparing the concentration of the at least two kynurenine metabolites in the sample to a range of values of the concentration of the at least two kynurenine metabolites for normal individuals, such that if the concentration of the at least two kynurenine metabolites in the sample lies outside of the range of values for normal individuals, epilepsy is diagnosed in the subject.

Preferably, the at least two metabolites are compared as a ratio selected from the group consisting of KA / 3HOAA, (KA+AA) /3HOAA, KA / QUIN, 3HOAA / 3HOKYN, KA/3HOAAxTRP, and (KA+AA)/3HOAAxTRP.

According to another embodiment of the present invention, there is provided a method for detecting a predisposition to epilepsy in a subject, the subject being substantially free of signs and symptoms of clinical epilepsy, the method comprising the steps of: (a) obtaining a sample from the subject; (b) measuring a concentration of at least two kynurenine metabolites, including at least one neuroprotective metabolite and at least one neurotoxic metabolite, in the sample; and (c) comparing the concentration of the at least two kynurenine metabolites in the sample to a range of values of the concentrations of the at least two kynurenine metabolites for normal individuals, such that if the concentrations of the at least two kynurenine metabolites in the sample lie outside of the range of values for normal individuals, the predisposition to epilepsy in the subject is detected.

According to yet another embodiment of the present invention, there is provided a method for determining an efficacy of treatment with an AED (antiepileptic drug) in a subject, comprising the steps of: (a) obtaining a sample from the subject; (b) measuring a concentration of at least two kynurenine metabolites in the sample including at least one neuroprotective metabolite and at least one neurotoxic metabolite; and (c) comparing the concentrations to an expected range of values for individuals with diagnosed epilepsy substantially controlled by treatment with an AED, such that the efficacy of treatment with

the AED in the subject is determined.

According to yet another embodiment of the present invention, there is provided a diagnostic system for diagnosing of epilepsy in a subject, comprising: (a) a sample taken from the subject; (b) a measurer for measuring a concentration of at least two kynurenine metabolites, including at least one neuroprotective metabolite and at least one neurotoxic metabolite, in the sample; and (c) a correlator for correlating the concentrations of the at least two kynurenine metabolites in the sample with a range of values for the ratios of the at least two metabolites for normal individuals, such that if the ratios of the at least two metabolites in the sample lie outside of the range of values for normal individuals, diagnosis of epilepsy in the subject is detected.

The following is a list of abbreviations which are used in the text:

AED - antiepileptic drugs;

CSF - cerebro-spinal fluid;

EP - epilepsy-prone;

ER – epilepsy-resistant;

TRP - tryptophan;

KYN - kynurenine;

KA - kynurenic acid;

AA - anthranilic acid;

3HOAA - 3-hydroxyanthranilic acid;

3HOKYN - 3-hydroxykynurenine;

QUIN - quinolinic acid;

3HAO - 3-hydroxyanthranilic acid oxygenase (EC 1.13.11.6);

IDO – indole-amine 2,3 – dioxygenase (EC.1.13.11.11);

NMDA - N-methyl-D-aspartate;

PL - pyridoxal;

PLP - pyridoxal 5-phosphate;

QPRT - quinolinic acid phosphoribosyltransferase (EC 3.2.2.5); and

KAT - kynurenine amino-transferase (EC 2.6.1.7).

The term "predisposition to epilepsy'' includes a predisposition towards seizures without the appearance of any clinical symptoms. Similarly, the term "clinical epilepsy''

includes the disease state in which seizures have become clinically detectable. Hereinafter, the term "sample" includes a portion of blood, urine or other body liquid or tissue removed from a subject for the purposes of diagnosis. The term "subject" preferably includes a human who is to be tested, but could also encompass an animal subject to be tested. The term "kynurenine metabolite(s)" or "kynurenines" includes all tryptophan metabolites formed within kynurenine pathway of tryptophan degradation up to Niacin: L-KYN, 3-HOKYN, 3HOAA, AA, KA, or QUIN. Hereinafter the term "anthranilates" includes AA and 3HOAA together.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIG. 1 is a graph of a portion of the kynurenine metabolic pathway;

FIG. 2 shows Table 1 for results of plasma kynurenines in epilepsy-prone (EP) rats and in rats with spontaneous non-convulsive absence seizures (GAER's), in comparison to epilepsy-resistant (ER) rats and the effect of anticonvulsive drugs, phenytoin (Pht) and ethosuximide (Ets), on plasma kynurenines;

FIGS. 3A and 3B are graphs for results concerning audiogenic convulsive reactions to treatment with phenytoin;

FIG. 4 shows Table 2 for brain kynurenines in EP rats in comparison with control ER and effect of phenytoin on brain kynurenine metabolites in EP rats;

FIG. 5 shows Table 3 for brain kynurenines in GAER's in comparison with ER rats and effect of ethosuximide on brain kynurenine metabolites in GAER's;

FIG. 6 shows Table 4 for plasma kynurenines in seizure-free epileptic patients and patients not controlled by antiepileptic drugs, in comparison with healthy children; and

FIG. 7 shows Table 5 of plasma kynurenines in children with repeated febrile convulsions in comparison with healthy children and children after the first episode of febrile convulsions.

DESCRIPTION OF THE INVENTION

The present invention is of a method for diagnosing clinical epilepsy and for detecting a predisposition to epilepsy, preferably substantially before clinical symptoms

become apparent. For diagnosing clinical epilepsy, the method of the present invention is particularly drawn towards clarification of the diagnosis of epilepsy. In addition, other provided embodiments include a method for optimization of a regimen for AED and diagnostic systems for performing the methods of the present invention.

The method of diagnosing epilepsy or of detecting a predisposition towards epilepsy of the present invention relies upon the altered balance of the concentrations of the metabolites of the kynurenine pathway of tryptophan degradation. This altered balance was found in animal models of genetic epilepsy and in patients with diagnosed epilepsy, both treated and non-treated by AED. Several underdiagnosed cases of epilepsy, as well as of individuals having a predisposition to epilepsy, were detected by using samples taken from the children of epileptic parents. Both clinical and experimental data obtained indicate that the changes in the plasma concentrations of the kynurenine metabolites correlate to the level of seizure predisposition, so that the concentrations of certain kynurenines are biochemical markers indicative of both a predisposition to epilepsy and of developed epilepsy. Under AED treatment some of the biochemical markers of seizure susceptibility are further altered, enabling their measurement to be used for an assessment of the efficacy of treatment.

Also, the concentrations of the kynurenine metabolites L-KYN, AA, 3HOKYN, 3HOAA, KA, xanthurenic acid and QUIN are indicative of the disorders in the absorption, transport or general metabolism of vitamin B6. Without wishing to be bound by a particular mechanism, one possible explanation for this correlation is that an inborn error in vitamin B6-metabolism may form a genetic basis for an enhanced susceptibility to seizures. Regardless of the particular mechanism, the altered concentrations of the metabolites of PLP-dependent kynurenine pathway can be used as the biochemical markers of the genetic predisposition to epilepsy, as shown in the Examples below. Furthermore, these parameters are altered during treatment with AED, so that in combination with the measurement of the concentrations of AED by any of variety of techniques used for measuring AED, these biochemical markers can be used for the effective monitoring of epilepsy therapy by AED.

The background art neither taught nor suggested that such biochemical markers were associated with epilepsy, and certainly did not teach or suggest that these markers could be used to detect a predisposition to genetic seizure disorders and/or the effectiveness of AED treatment in a subject. Furthermore, the background art certainly did not teach or suggest non-invasive methods for a biochemical approach to the diagnosis of epilepsy or of the

detection of a predisposition towards epilepsy, such as the analysis of blood plasma, urine or other body liquids and tissues.

Under AED treatment these markers can actually be used for the evaluation of the effectiveness of AED treatment and/or AED overdosing, which was also neither taught nor suggested by the background art.

These methods and diagnostic systems of the present invention could beneficially be used to screen several groups of high-risk individuals. The following groups of individuals at high risk for development of seizure disorders should preferably be tested, including, but not limited to, the first and second degree relatives of patients with epilepsy; babies born through a traumatic or premature delivery; children with febrile convulsions and attention deficit disorders; patients who have endured brain trauma or intracranial infections; pregnant women; and soldiers and pilots who may be subjected to stressful situations. In addition, epileptic patients treated with AED should preferably be regularly and repeatedly tested, especially those patients who are not being treated effectively.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of a method for diagnosing clinical epilepsy, especially for clarification of an existing diagnosis of the disease, and of detecting a predisposition to epilepsy, preferably substantially before clinical symptoms become apparent. In addition, other provided embodiments include a method for determining an optimal regime for AED treatment in a subject and diagnostic systems for performing the methods of the present invention.

The method of detecting a predisposition to epilepsy and of clarifying the diagnosis of clinical epilepsy by a biochemical approach according to the present invention relies upon the altered balance of the metabolites of the kynurenine pathway of tryptophan degradation. As further described in the Examples below, the tryptophan metabolites of the kynurenic pathway were examined in the plasma and brain of two animal models of genetic epilepsy, i.e., of seizure-naive genetically epilepsy-prone (EP) rats and rats with spontaneous non-convulsive absence seizures (GAERs) , in comparison with the metabolites in the plasma and brain of control epilepsy-resistant (ER) rats. Also plasma samples of patients with epilepsy, as well as of individuals under pre-epileptic condition, were studied in comparison with

samples obtained from healthy individuals. These experiments showed that the correlation between the plasma levels of kynurenines and the level of seizure susceptibility existed both in animal models of genetic epilepsy and in clinical materials. Characteristically increased levels of anthranilates, (AA+3HOAA), and an enhanced ratio of anthranilates to their common precursor KYN (AA/KYN, 3-HOAA/KYN), were found both in seizure-naive EP animals and in epileptic patients. Also characteristically decreased levels of KA and in the ratio KA/3HOAA were found both in animal models of epilepsy and in untreated or non-effectively treated epileptic patients, in comparison with healthy or effectively treated patients. These altered ratios enable the detection of a predisposition to epilepsy and the clarification of the diagnosis of epilepsy according to the methods of the present invention.

As an index of seizure predisposition, the ratio of the concentrations of two functionally opposite metabolites, neuroprotective KA to neurotoxic 3HOAA, are especially preferred as the biochemical markers for determining the efficacy of AED treatment.

The importance of these markers is emphasized by the results of the animal experiments described below, in which 3-HOAA deficiency was found in certain regions of the epileptic brain. In addition, the animal models were used to study the effects of both acute and chronic AED administration on kynurenine metabolism. Without wishing to be bound by a particular mechanism, the results obtained suggest that pyridoxine imbalance in the plasma and brain may underlie an increased seizure susceptibility.

Section I. Animal Experiments

Example 1

Plasma Levels of Kynurenine Metabolites in Seizure-naive Epilepsy-prone Rats and Epilepsy-resistant Rats

Audiogenic sensitive EP rats as the animal model of genetic epilepsy were used in comparison with control ER rats. Both strains were developed by, and are available from, Dr. Ch. Marescaux at the Strasbourg UNISERM 398 laboratory.

The plasma levels of the kynurenine metabolites were measured in both groups of rats. Results are given in Tables 1-3. The methods were as follows.

Methods.

The animals were sacrificed by guillotine. Blood (1.5 ml) was collected in the tubes.

After centrifugation (2000 g x10 min at the room temperature) plasma and erythrocytes were kept separately under -80° until processing.

Plasma concentrations of TRP and related metabolites, L-KYN, KA, AA, 3HOKYN and 3HOAA, were measured by the HPLC (High Pressure Liquid Chromatography) method. The reference standards of the metabolites which were measured in the samples (L-TRP, L-KYN, AA, 3HOKYN, 3HOAA and KA, as well as zinc acetate) were purchased from Sigma (USA). Acetonitrile, acetic and perchloric acids were obtained from BioLab (Israel).

Millimolar stock solutions of each standard were prepared and stored at -80°. Blood samples taken from each animal were deproteinized by addition of 100 microliters of 2.4 M perchloric acid to 0.5 ml of plasma. After centrifugation (10 000 g, 10 min), the supernatants were filtered (0.22 mmc filter from Millipore).

Specifically, quantitative determinations were obtained using a LaChrom HPLC system (Merck-Hitachi) which includes: LaChrom L-7100 HPLC-Pump, LaChrom L-7200 Autosampler, LaChrom L-7400 UV-VIS Detector , LaChrom L-7480 Fluorescence Detector, LaChrom D-7000 Interface module and LaChrom D-7000 Multi HPLC System Manager Software. The analytical column was a 250 mm x 4 mm I.D. column, packed with Lichrospher 100 RP-18 (5mmc) (Merck, Germany).

100 microliters of each sample were injected into the column. The mobile phase consisted of 50 mM acetic acid, 250 mM zinc acetate (pH 4.9) with 2% (v/v) acetonitrile. The concentration of each metabolite in the sample was determined as the area under the peak, and was corrected by reference to the values obtained for the reference metabolites. Separations were achieved at ambient temperature by isocratic elution at a flow-rate of 1.0 ml/min. The detection and the quantitation were carried out with the fluorescence detector connected on line with a UV Detector. The fluorescence excitation and emission wavelengths were set to 320 nm and 420 nm (respectively) at the beginning of the run. Twenty minutes later, excitation and emission wavelengths were changed to 254 nm and 404 nm. UV signals were then monitored at 365 nm. The results were as follows.

Results

The experiments showed that in the plasma of seizure-naive EP rats, the levels of KYN and KA were lower, while the levels of both anthranilates, AA and 3HOAA, were higher, than in the plasma of ER rats.

The results presented in Table 1 (Figure 2) show that in addition to having a significantly higher level of plasma tryptophan, EP animals also have much lower levels of KYN, a KA precursor, and KA itself. Accordingly, lower ratios of KYN to TRP and of KA to TRP have been found in EP animals as compared to control ER rats.

The deficiency of neuroprotective KA becomes even more striking, when the level of KA is correlated to the level of neurotoxic 3HOAA, which in EP rats is almost twice higher than in normal rats. The ratio of these functionally opposite metabolites (KA/3HOAA) in EP animals is approximately 38 fold lower than in control ER rats. As an index of seizure susceptibility, this parameter clearly discriminates between seizure-naive EP animals and control ER ones.

Unlike KA, the concentration of AA, another neuroprotective metabolite (Jhamandas et al, 1990), is more than twice as high in EP rats than in control ER rats, while the ratio of AA to KYN is more than 12 fold higher in EP rats than in ER rats.

The total sum of both anthranilates, 3HOAA and AA, is also twice as high in EP rats than in ER animals. The difference between these results in EP and ER rats becomes especially impressive when the total sum of both anthranilates is correlated to the level of KYN, and when the level of 3HOAA and AA is separately correlated to the level of KYN, the common substrate of both products. Given the increased levels of the anthranilates along with the deficient level of KYN, the value of each of these ratios 3HOAA/KYN, AA/KYN and (AA+3HOAA)/KYN in EP animals is 12-13 times more than in ER rats.

Thus, these indices which reflect an intensity of kynureninase reaction provide evidence for much higher kynureninase activity in seizure-naive genetically EP rats, than in control ER animals. The constellation of tryptophan metabolites of kynurenine pathway found in the blood plasma provides biochemical markers for distinguishing potentially epileptic animals from normal ones.

Taken together, the lower level of neuroprotective KA and especially the lower ratio of KA to 3HOAA, combined with the increased levels of both anthranilates and the low concentration of their common precursor KYN, characterize the kynurenine profile for epilepsy predisposition.

Example 2

Plasma Levels of Kynurenine Metabolites in Rats with Spontaneous Non-convulsive Absence Seizures (GAERs)

To detect the levels of kynurenines in the plasma of rats with spontaneous non-convulsive absence seizures (GAERs), the same method was used as in Example 1 above. In GAERs, constant absence seizures were seen as arrest behavior accompanied by the characteristic spine-and-wave EEG activity (up to 800-900 sec/hour).

As well as in EP rats, the levels of KYN and KA in GAERs were strongly lower than in control ER rats. However, unlike EP animals, the plasma level of neuroprotective AA was also diminished in them (Table 1, Figure 2). As well as in EP animals, the levels of both 3HOAA and AA correlated to the level of KYN were significantly (2.0-4 times) higher in GAERs than in control ER rats. As in EP rats, the ratios of KA to 3HOAA and of (AA+KA) to 3HOAA in GAERs were significantly lower than in control ER rats (Table 1, Figure 2).

Thus, the results obtained indicated that seizure-naive genetically EP animals and animals with absence epilepsy (GAERs) could be differentiated from control genetically ER rats, according to the plasma pattern of kynurenine metabolite levels. The lower plasma levels of KYN and KA, together with the lower ratios of KA/3HOAA and the increased ratios of 3HOAA and AA to KYN were shown to be indicative for the enhanced seizure susceptibility. Moreover, the type of seizure predisposition or/and the type of seizures could be detected by the pattern of the plasma kynurenine metabolite levels.

Example 3

Monitoring of Antiepileptic Treatment with Plasma Kynurenines in Animal Models of Genetic Epilepsy

Since the suggested diagnostic biochemical markers were shown to be suitable for detecting the level of seizure susceptibility, as shown in Examples 1 and 2, their ability to assess the efficacy of AED treatment was examined. The experiments were carried out in the same animal models of genetic epilepsy as in Examples 1 and 2, that is, in audiogenic sensitive EP rats and in rats with spontaneous absence seizures (GAERs).

Monitoring of Phenytoin Treatment According to the Levels of Plasma Kynurenine Metabolites in Epilepsy-prone Rats

The intensity of sound-induced seizures audiogenic sensitive EP rats before and after AED treatment was comparatively assessed in the same group of EP animals and then correlated with the levels of the kynurenine biochemical markers.

Audiogenic sensitive EP rats react to the intensive sound stimulation 105-120 dB with a running stage ("wild running"), followed by generalized tonic-clonic convulsions. Depending on the severity of the reaction, the convulsions may end with cataleptic immobility, post-ictal excitation, or coma. The anticonvulsive effect of phenytoin, the AED, which is clinically administrated for treatment of generalized tonic-clonic convulsions, was shown to be correlated with the biochemical markers of seizure susceptibility according to the present invention.

Eleven audiogenic sensitive EP rats and eleven control ER rats were used in experiments at the age of 2.5 months. When subjected to the audiogenic stimulation (110 db, 30 sec), all 11 audiogenic-sensitive EP rats developed severe generalized tonic-clonic convulsions, ended by coma (in 6 animals), or post-ictal excitation (5 rats). Estimated in scores, the intensity of audiogenic convulsions in this group of animals was 10.5 (see Figures 9 and 10). The scores were estimated as follows:

0 - absence of reactions
1 -short-term running stage
2 -two-phase running stage
4 -tonic convulsions of flexors
6 -tonic convulsions of extensors
8 - postictal excitation or muscle atonia
10 - postictal excitation with convulsions
12 - postictal coma
16 – death

The mean duration of the stages of audiogenic attacks in EP rats is shown in Figure 3A. No sound-induced convulsive reactions were observed in the control ER group.

Starting the following day, the EP animals were injected with phenytoin. The

treatment protocol included a first injection of phenytoin 75 mg/kg i.p., followed by 12 successive injections of phenytoin (50 mg/kg) once per day (Loscher et al., 1985).

After 13 days of phenytoin treatment, the incidence and intensity of sound-induced convulsions were estimated again. Each animal was tested on day 13 and again on day 14. Altogether 22 tests of sound stimulation were performed. The patterns of the audiogenic reactions observed before and after chronic phenytoin treatment were compared (see Figures 3A and 3B). Phenytoin administered according to the above protocol provided a protective effect against sound-induced generalized tonic-clonic convulsions. Sixteen out of the twenty-two tests of sound stimulation did not induce any convulsions, so that only six tests resulted in sound-induced convulsions. The intensity of these six sound-induced convulsive reactions was significantly reduced: the duration of tonic convulsions (the most severe stage of the attack) was remarkably shorter, while the duration of the latency and running stage phases (the least traumatic stages of a sound-induced convulsive attack) were longer, than in non-treated EP rats. There were neither post-ictal excitation, nor coma. Furthermore, cataleptic immobility arose at the moment when the sound ceased, or even preceded this moment, so that estimated in scores the intensity of audiogenic convulsions after Phenytoin treatment was 4.1 (see Figures 3A and 3B).

The next day, eight animals were selected which did not convulsively react to the sound stimulation when repeated twice, showing that these EP rats were completely protected by chronic phenytoin treatment. These eight selected animals were sacrificed, and blood samples were taken, processed and analyzed as described above for Example 1.

A significant decrease in the level of TRP with an appropriate increase in the levels of KYN - up to the values characteristic of control ER animals -, and especially of neuroprotective KA, characterize the effect of the phenytoin treatment in EP animals. The level of KA in phenytoin treated EP animals turned out to be 13 fold higher than in non-treated EP rats and 1.43 fold higher than in the control ER rats (see Figure 4). Also, the ratio of KA to TRP in phenytoin treated animals was strongly higher than in the control ER rats. In addition, the concentration of neurotoxic 3HOAA in phenytoin-treated rats was a bit lower than in non-treated EP rats and almost equal to the level found in control ER rats (Figure 4). The opposing trends of the concentrations of these two functionally opposite metabolites resulted in a dramatic increase in the mean value of KA/3HOAA ratio under phenytoin treatment. This ratio in phenytoin treated EP rats was about 35 fold higher than in non-

treated EP rats, and was equal to the mean value found in the control ER rats (see Figure 4).

Thus, under AED treatment, the plasma ratio KA/3-HOAA was shown to be correlated with the level of seizure susceptibility. The increased value of this ratio corresponded to the lower level of seizure predisposition achieved from the regular administration of the AED phenytoin. The increased value of the ratio of the total sum of both protective compounds (KA+AA) to 3-HOAA (Figure 4) was also indicative of the lower level of seizure predisposition.

Moreover, AED overdosing can be diagnosed by the suggested markers. A striking excess of both KA and of the ratios KA/TRP and KA/3HOAA in some of phenytoin-treated EP animals was found to be indicative for phenytoin overdose during the course of treatment. For example, the observed concentrations of KA at 875.7 nM and 1341.4 nM produced corresponding KA/3HOAA ratios equal to 101.8 and 72.1 respectively, which were significantly higher than the equivalent measurements characteristic of control ER rats, and indicated phenytoin overdosing. Some other observed symptoms, such as hair loss, impaired motor co-ordination, hard (stone-like) liver, regional bleeding in the lungs, and enlarged adrenal glands were found and pointed to phenytoin overdosing (data not shown). By contrast, concentrations of KA at 222.6 nM and 223.5 nM produced corresponding KA/3HOAA ratios equal to 15.9 and 15.5 respectively, were at the end of the range of values observed for normal animals and indicated that the individual dose of AED could be increased.

Thus, the experiments in EP rats have shown that the measurements of the ratios KA/3HOAA or (KA+AA)/3HOAA, and KA/TRP, in parallel with the concentrations of AED in plasma, which are routinely measured by a variety of techniques, are indicative of effective or ineffective AED treatment.

Monitoring of Ethosuximide Treatment with Plasma Kynurenines in Rats with Spontaneous Non-convulsive Absence Seizures (GAERs)

Ethosuximide (Ets), which selectively prevents absence seizures, was tested under two different conditions: single administration (70 mg/kg p.o. (oral)) and chronic administration (40 mg/kg p.o. twice a day over the period of 20 days) which attempted to induce an overdose of the AED.

After the administration of a single dose of ethosuximide (70mg/kg), the drug

effectively blocked spike-and-wave EEG activity and prevented characteristic arrest behavior almost completely. The striking increase in the plasma concentrations of both neuroprotective compounds, AA and KA, with a corresponding increase in the ratios of KA/3-HOAA and (KA+AA)/3-HOAA, correlated to the observed therapeutic effect of ethosuximide (Table 1, Figure 2).

The chronic administration of ethosuximide, and the resultant overdose of this drug, resulted in a increase in the frequency and duration of absence seizures and correspondingly of spike-and-wave EEG seizure activity up to 1200-1600 sec/hour. A decrease in the plasma levels of both neuroprotective compounds, KA and AA, was seen, down to the initial levels found in untreated GAERs. In addition, the level of neurotoxic 3-HOAA increased in response to administering an overdose of ethosuximide. Also the ratios of KA/3-HOAA and (KA+AA)/3-HOAA decreased to levels which were lower than those found in untreated GAERs were found (Table 1, Figure 2).

Hence, the model experiments in GAERs have shown that both a protective therapeutic effect from AED administration and the effects of administering an overdose of AED can be detected by the suggested markers, which include the plasma concentrations of the kynurenine metabolites and the ratios especially the ratios between the neuroprotective (KA,AA) and neurotoxic (3HOAA) metabolites..

Analysis of Brain Kynurenines in Animal Models of Genetic Epilepsy and the Effect of Antiepileptic Treatment on Brain Kynurenines

The analysis of the levels of kynurenines in the brain was performed in the same animal models of genetic epilepsy as in Examples 1 and 2 above, i.e., in seizure-naive EP rats and rats with spontaneous non-convulsive absence seizures (GAERs), in comparison with control ER rats. In addition, the level of brain kynurenines was determined in EP rats chronically treated with phenytoin) and in GAERs treated by specific AED Ets under different treatment protocol (as in Example 3 above). The experimental method was as follows.

The animals were sacrificed at the age of 2.5 months. Brain tissue was dissected on a cold plate. The samples of the cortex, brain stem , midbrain and cerebellum were collected and immediately frozen for storage at -80 °C. The homogenized brain samples were prepared for HPLC detection and analyzed as previously described. The results are shown in Table 2, Figure 4 and Table 3, and Figure 5

Example 4.

Brain Kynurenines in Epilepsy-prone Rats:

Effect of Phenytoin

Three groups of animals were used for the comparative analysis of brain kynurenines: seizure-naive EP rats; EP rats chronically treated with phenytoin according to the protocol described in Example 2 (as in Example 2, only those animals which were completely protected by phenytoin treatment against sound-induced convulsions were used for the analysis of brain kynurenines); and control ER rats.

As seen in Tables 1 and 2 (Figures 2 and 4, respectively), the concentrations of kynurenines in the brain were significantly different from the concentrations of these metabolites in the plasma. Moreover, the level of each kynurenine metabolite was rather specific for each brain area examined (Table 2, Figure 4).

Only TRP and 3HOAA were consistently detected in all brain regions, both in ER and in EP animals, while the amounts of other compounds, including KYN, 3-HOKYN , AA and KA, varied in EP and ER animals from non-detectable up to significant concentrations.

No difference was found in the brain levels of TRP in EP and ER rats. Neither KA,

nor AA were found in the brain of ER rats, while these neuroprotective compounds were detected (though inconsistently) in all brain regions of EP rats (Table 2, Figure 4).

The concentrations of 3HOAA in the cerebellum, the main inhibitory brain structure, were found to be significantly different in EP and ER animals. The level of 3HOAA in the cerebellum of EP animals was 3 fold lower, than in the cerebellum of ER rats. The value of the ratio of 3-HOAA/TRP, indicating the level of 3-HOAA related to the concentration of TRP, confirms the significant deficiency of 3HOAA production in the cerebellum of EP rats (Table 2, Figure 4).

Decreased 3-HOAA synthesis was also found in the midbrain and brain stem of EP rats. The total concentration of 3HOAA in the brain stem of EP rats was about 30% lower than in ER rats, though the level of 3HOAA related to the concentration of TRP (3-HOAA/TRP ratio) was equal in both groups of animals. On the contrary, in the midbrain, the ratio of 3-HOAA to TRP in EP rats was found to be lower than in ER animals, regardless of the equal concentrations of 3-HOAA in the region of both groups (Table 2, Figure 4).

In accordance with the deficiency of 3-HOAA in the cerebellum and the brain stem , the concentrations of 3-HOKYN, the precursor of 3-HOAA, in these two regions of EP rats was shown to be 70-100 fold higher than the amount of this compound detected in normal subjects by Gal & Sherman (1980).

Unlike other brain structures examined, about 40% of the excess amount of 3-HOAA, with the corresponding increase in the 3-HOAA/TRP ratio, was found in the cortex of EP rats in comparison with ER animals. Along with the increased level of 3-HOAA, the highest amounts of both protective compounds, KA and AA, were detected in the cortex of EP animals. The inventors believe that the specific peculiarities of kynurenine metabolism detected in the cortex of EP rats may be considered as a distinctive biochemical feature of the cortical metabolism found in EP animals (Dolina a. Kozak, 1987; Vriend et al., 1991; Dolina et al., 1993).

Chronic administration of phenytoin according to the treatment protocol described in the Example 3 resulted in restoration of the cerebellar 3-HOAA level, up to the level characteristic of control ER rats. A significant increase in the concentration of KA was found in the brain stem (Table 2, Figure 4). Excitation of this specific brain region results in the tonic stage of general convulsions. Without wishing to be bound by a single mechanism, the

biochemical correction of the cerebellar inhibitory function, together with an increase in the level of neuroprotective KA in the brain stem, may contribute to the inhibition of audiogenic generalized tonic-clonic convulsions provided by phenytoin and described above (Example 3).

In conclusion, the experiments on this model of genetic epilepsy have shown that the particular constellation of brain kynurenines correlates to genetic predisposition to generalized convulsive attacks. This constellation is specifically changed under AED treatment aimed at the prevention of generalized tonic-clonic convulsions.

Example 5

Brain Kynurenines in Rats with Spontaneous Non-convulsive Absence Seizures(GAERs): Effect of Ethosuximide

The experiment results show that the disordered brain kynurenine metabolite levels in GAERs, with their distinct form of epilepsy, are localized differently from those in EP animals predisposed genetically to generalized tonic-clonic convulsions. A pronounced 3HOAA deficiency was found in the midbrain and cerebellum of GAERs, while an excess of 3HOAA of 70% above the cortical level characteristic of ER rats was revealed in the cortex (Table 3, Figure 5).

Ethosuximide, which selectively prevents absence seizures, was tested in two different treatment protocols. Under single p.o. administration, ethosuximide (70mg/kg) effectively blocked spike-and-wave EEG activity and prevented characteristic arrest behavior almost completely. The therapeutic effect of ethosuximide correlated with the strong increase in the level of neuroprotective AA in the midbrain, although AA was not detected in this region in untreated GAERs. In addition, this therapeutic effect was correlated with the decrease in the cortical concentration of 3HOAA to the levels characteristic of control ER rats.

By contrast, the chronic administration of ethosuximide at the dosage 40 mg/kg p.o. twice a day over the period of 20 days (as in the Example 3 above), thereby causing an overdose, resulted in exacerbation of the symptoms of epilepsy, including an increase in the frequency and duration of periods of arrest behavior accompanied by an increase in the duration of spike-and-wave EEG seizure activity up to 1200-1600 sec/hour. The pro-

convulsive effect of an overdose of ethosuximide correlated with the disappearance of AA from the midbrain and with an increase in the cerebellar level of 3HOAA up to a level which was even higher than in untreated GAERs.

Thus, the experiments carried out in two animal models of genetic epilepsy have shown the correlation between the pattern of the brain kynurenine metabolites levels and the type of seizure activity (or genetic predisposition to this specific type of seizure activity). The levels of the brain kynurenine metabolites and their specific regional distribution permit the assessment of the efficacy, or lack thereof, of AED therapy.

Moreover, achieving a balance of kynurenine metabolites is suggested as a novel target for the development of AED therapy, rather than simply increasing the seizure threshold.

Section II. Clinical study

Example 6

Plasma kynurenines in children with Febrile Convulsions: condition at risk of developing epilepsy

It is known that febrile convulsions (FC) are mutually linked with epilepsy. FC increases the risk of developing epilepsy (Annegers et al., 1987; Wolf a. Forsythe, 1989; Lee, 1989). According to general population studies, 7% of children with FC will have epilepsy by the age of 25 years. Repeated FC are considered in the literature to be a pre-epileptic condition. On the other hand, an increased incidence of FC was shown in relatives of children with FC, with the frequency in first degree relatives varying from 7% to 31% (Lennox-Buchtal, 1973; Tsuboi 1987; Lee, 1989). Children with FC were therefore separated into three groups: a group with repeated FC, a group of FC children belonging to families with a history of FC , or epilepsy, and a group of FC children without such a genetic burden. The blood samples were taken within 24 hours after the episode of FC and processed as described above for Example 1. The data obtained were compared with the results obtained in healthy children (Table 4, Figure 6).

As shown from the data obtained, the plasma level of neurotoxic 3-HOAA in children with repeated FC is significantly higher than in healthy children, while the ratio of KA to 3-HOAA and this ratio calculated relative to the level of TRP are the lowest in this

group compared to all others. A similar tendency is found if the group of FC children with a family history of epilepsy is compared to a group without such a history: even after the first episode of FC, the level of neuroprotective AA and the total blood level of both neuroprotective compounds (AA+KA) are lower in the first group as compared to the second group. Also the balance between neuroprotective and neurotoxic compounds, measured by the ratio (AA+KA)/3HOAA, tends to be lower in children from families with a history of epilepsy or FC, than in FC patients without such a history, after the first febrile convulsive episode (Table 4, Figure 6). Hence, the imbalance between the concentrations of neuroprotective and neurotoxic compounds is of predictive value, since the individuals at risk of development of epilepsy can be diagnosed according to these parameters.

Example 7

Monitoring of AED therapy

To evaluate whether the suggested diagnostic markers can be used for monitoring of AED, the parameters of kynurenine metabolism were compared in epileptic children who were seizure free under administered AED therapy (i.e., well controlled by AED), and in children who had repeated epileptic attacks in spite of the administered treatment (i.e., non-controlled by AED). The patients were considered to be well controlled if they were seizure free for more than three months.

As shown by the results obtained (Table 5, Figure 7), there were no differences in the levels of TRP and KYN between epileptic children who were well controlled by AED and healthy children. The levels of KA and (KA+AA) in well controlled epileptic children were significantly higher than in the control group of healthy children. By contrast to the neuroprotective compounds, the level of 3HOAA was slightly decreased in seizure free children, in comparison with the healthy group, so that the ratio KA /3-HOAA and the ratio (KA+AA)/3HOAA was about 2 fold higher in the group of well controlled children, than in healthy group. The correlation of the ratios KA/3HOAA and (KA+AA)/3HOAA to the level of TRP corroborates the observation: the values of the ratio (KA/3HOAA)/TRP and of the ratio (KA+AA)/TRP for the group of seizure free children were twice as high than the value of these parameters for healthy children (Table 5, Figure 7).

In comparison with both the healthy group and the group of seizure free children,

decreased levels of TRP, KYN and especially of 3HOAA were found in the plasma of epileptic children non-controlled by AED. Such a decrease in the level of 3-HOAA results in higher ratios of KA/3HOAA and (KA+AA)/3HOAA in patients who are non-controlled by AED, in comparison with the group of seizure free patients. When correlated to the level of TRP, both ratios, KA/3HOAA and (KA+AA)/3HOAA, were twice as high in the non-controlled group as in the group of seizure free patients, and more than three times as high as in the healthy group (see Table 5, Figure 7). Without wising to be bound by a single mechanism, these data may indicate that the cumulative effect of several AED resulted in alterations of the activity of enzymes of the kynurenine pathway, mostly, in an inhibition in kynureninase forming 3HOAA, along with an over-activation of IDO, which causes a paradoxical increase in these ratios without parallel clinical success.

Thus, reflecting the level of seizure predisposition, the ratio of KA/3-HOAA enables the effectiveness (or non-effectiveness) of administered AED treatment in epileptic patients to be assessed.

In conclusion, the examples presented support a correlation between the balance of kynurenines in the blood and brain and propensity to epileptic seizures. These parameters are sensitive to AED treatment and are therefore helpful for choosing a correct therapeutic strategy. Based on these quantitative parameters, the diagnostic system is aimed at the detection of predisposition to epilepsy and the improved monitoring of AED therapy.

Example 8

Methods and Diagnostic Systems for Diagnosis of Epilepsy or Predisposition to Disease and for Monitoring of Antiepileptic Treatment

From the biochemical markers found and evaluated in the Examples above, methods and diagnostic systems for the diagnosis of both clinical and pre-clinical epilepsy can be made. In particular, the diagnosis of clinical epilepsy could be clarified with the methods and diagnostic systems of the present invention. A predisposition for epilepsy could also be detected.

The methods would involve the measurement of at least one kynurenine metabolite, and more preferably at least two kynurenine metabolites, in the sample taken from the subject. The following discussion centers upon a description of the analysis of two such metabolites in the sample as the preferred embodiment. In one embodiment, the method

involves the following steps. First, the sample is obtained from the subject. Preferably, the sample is a blood sample, which could be withdrawn with a needle, for example, according to well known procedures in the art. Alternatively and preferably, the sample is a urine sample, although any other fluid or tissue sample could be used. Next, the level of at least two kynurenine metabolites in the sample would be measured. Preferably, the tested metabolites at least include metabolites selected from the group consisting of KYN, 3HOAA, 3HOKYN, AA, KA and QUIN. More preferably, the concentrations of two metabolites are measured, either KA and 3HOAA , or the anthranilates AA and 3HOAA, or KYN and 3HOKYN, or 3HOAA and QUIN. Also more preferably the ratio of the concentrations of at least two metabolites is measured, such as the ratio of the concentration of KA to the concentration of 3HOAA, or the ratio of the total concentrations of AA and 3HOAA (AA+3HOAA) to the concentration of KYN ((AA+3HOAA)/KYN). Most preferably, the concentrations of functionally opposite metabolites KA and 3HOAA (KA/3HOAA), or the ratio (KA+AA)/3HOAA, or KA to QUIN or (KA+AA)/QUIN are detected.

As described in further detail below, the level could be measured through HPLC, fluorimetry, or an immunological test such as an ELISA, for example. The measured level would then be compared to the range of values for normal individuals without epilepsy.

According to another embodiment, the method of the present invention could be used to determine the efficacy of AED treatment. First, the sample would be obtained from the subject as previously described. Next, the level of at least one kynurenine metabolite would be measured. Preferably, the metabolite would be selected from the group consisting of TRP, KYN, KA, AA and 3HOAA . More preferably, a ratio of the concentrations of at least two metabolites, such as the ratio of the concentration of KA to the concentration of 3HOAA, or the ratio of the concentration of KA+AA to the 3HOAA would be measured. Next, the ratio would be preferably compared to the range of values obtained for normal subjects, and optionally to the range of values obtained for subjects with non-controlled epilepsy and subjects with well-controlled epilepsy, in order to assign the tested subject to one of these three groups and hence to make the diagnosis of successfully controlled or non-successfully controlled epilepsy.

As noted above, the successful treatment of epilepsy with AED should result in an increase in KA/3HOAA ratio which is about two fold higher than in healthy individuals. For

individual metabolites, in subjects with successfully controlled epilepsy the concentration of KA should be about 1.5-2 fold higher, while the concentration of 3-HOAA should not be reduced by more than 1.5-2 fold as compared to normal individuals.

Preferably, each of these measurements of the ratio of the kynurenines of opposite properties ,or a set of kynurenine metabolites, is to be correlated with a measurement of an AED level (by any of regularly used technique) in a sample of the subject, more preferably the same sample of the subject. This correlation could be used to determine whether the amount of AED being administered should be altered by being increased or decreased, for example, or whether a different AED should be administered.

Another embodiment of the present invention would be a diagnostic system for implementing the methods of the present invention. The system would include a measurer for measuring a concentration of at least one kynurenine metabolite, but more preferably for measuring the concentrations of a set of functionally opposite metabolites (KA and 3HOAA; or KA, AA and 3HOAA) in a sample taken from a subject, substantially as described for the methods above. The term "functionally opposite" refers to a group of metabolites which includes at least one neuroprotective metabolite and at least one neurotoxic metabolite. The system would also preferably include a correlator for correlating the measured concentration with a range of values of this concentration observed in normal subjects. The correlator could be a software program written in a computer language such as C or C++ and operated by a computer, for example. One of ordinary skill in the art could easily construct such a software program.

A number of different technical approaches to the measurer of the diagnostic system are possible. As described in the previous Examples, the first approach is to use HPLC to analyze the blood samples, or other samples taken from patients. However, this approach can be technically difficult to implement on a wide scale. Therefore, one of three other approaches could also be used. It should be noted that these are intended as examples only and are not meant to be limiting.

Spectrofluorimetric Measurement of Anthranilates (AA+3HOAA) and KA in the Same Sample

The difference in the spectra of fluorescence of anthranilates and kynurenic acid permits discrimination between them in the same sample, even if HPLC is not used to

separate these metabolites. While for anthranilates the optimal excitation and emission wavelengths are at 320 nm and at 420 nm (respectively), these parameters for KA are 254 and 404 nm (respectively). Furthermore, both AA and KA fluoresce only in the presence of an additional molecule, zinc salts in these experiments, while the other metabolites analyzed in the same sample using HPLC with a fluorimetric detector either do not fluoresce (such as L-KYN and 3-hydroxykynurenine), or do not fluoresce in the absence of the additional molecule.

Therefore, the measurements of the parameters of fluorescence in the presence or absence of the specific helper molecule permit separate detection of the total concentrations of anthranilates (AA+ 3HOAA) and KA.

Activity of Key Enzymes of the Kynurenine Pathway

The concentrations of metabolites depend on the activity of the key enzymes of the metabolic pathway. The rate limiting enzymes of the kynurenine pathway are kynureninase, kynurenine-3-hydroxylase, 3-hydroxyanthranilic acid-dioxygenase and QUIN phosphoribosyl-transferase (QPRT). The activity of the rate limiting enzymes of the kynurenine pathway can be used for diagnosis of a predisposition to epilepsy, for clarification of the diagnostics of the disease, and for monitoring of AED treatment.

Some of these enzymes are found in the blood cells: kynureninase activity has been detected in lymphocytes, while QPRT activity was found in erythrocytes. Therefore, homogenates of lymphocytes can be used for further detection of the activity of kynureninase, while homogenates of erythrocytes can be used for detection of QPRT activity. In addition, the activity of alkaline phosphatase, which may be involved in the defect in the metabolism of kynurenines, can also be measured in peripheral tissues such as blood cells.

Immunochemical Detection

Immunochemical detection is based on a highly specific antigen-antibody reaction. Such detection is highly effective and reliable in operation. Several steps should be performed to develop suitable assays for immunochemical detection. First, an antigen must be developed. Since the kynurenines are low molecular weight compounds, they are preferably linked as haptens to compounds of high molecular weight (such as a protein,

polysaccharide, or a synthetic polymer) to prepare "anti-kynurenine" antibodies, as is well known in the art. For example, the kynurenines could be linked to keyhole limpet hemocyanin as a protein carrier for immunization. Next, animals are immunized to develop highly specific antisera. Optimal antisera are then preferably selected. Finally, the conditions for the reaction are then preferably optimized, including time, temperature, concentrations of reagents and the method to detect the antigen-antibody (such as ELISA). Both polyclonal and monoclonal antibodies can be used for the development of these assays, as can other immunologically reactive molecules such as Fab' fragments.

Once the assay has been developed, the evaluation of the effectiveness of the assay for the pre-clinical assessment of seizure predisposition is performed. The blood samples are compared to the samples taken from control non-epileptic patients.

It will be appreciated that the above descriptions are intended only to serve as examples, and that many other embodiments are possible within the spirit and the scope of the present invention.

APPENDIX

REFERENCES

Aicardi J. *Epilepsy in children.* 2nd edition. New York: Raven Press, 1994: 18-43.

Andermann E. Multifactorial inheritance of generalized and focal epilepsy. In: Anderson VE, Hauser WA, Penry JK, Sing CF, ed. *Genetic Basis of the Epilepsies*. New York: Raven Press 1982:355-74.

Anderson EV, Hauser WA. Genetics. In: Dam M, Gram L, ed. *Comprehensive Epileptology*. New York: Raven Press 1990:57-76.

Bird TD. Genetic considerations in childhood epilepsy. *Epilepsia* 1987; 28(suppl.1):71-81.

De Fendis FV. Studies on tryptophan loading - an overview. *Drugs of today* 1988; 24 (4): 257-68.

Deldago-Escueta AV, Treiman DM, Enrile-Bascal F. Phenotypic variations of seizures in adolescent and adults. In: *Genetic Basis of the Epilepsies*. New York: Raven Press 1982:49-81.

Dolina S, Kozak H. Is B6 dependency an essential factor of genetic predisposition to epilepsy? In: *17-th Epilepsy International Congress Book of Abstracts*, Jerusalem 1987:34.

Dolina S, Keller A, Kozak A. Genetic vitamin B6 dependency as a determinant of epileptic diathesis. In: *19th Annual Meeting Society for Neuroscience* 1989, October, Phoenix, Arizona.

Dolina S. Is pyridoxine dependency an inborn error of metabolism triggering an enhanced convulsibility in different animak models of genetic epilepsy? A commentary. *Neurosci. (Kobe)* 1992; 18 supplement 2:153-62.

Dolina S, Peeling J, Sutherland G, Pillay N, Greenberg A. Effect of sustained pyridoxine treatment on seizure susceptibility and regional brain amino acid levels in genetically epilepsy-prone BALB/c mice. *Epilepsia* 1993; 34:33-42.

Dykens JA, et al. Oxidative reactivity of the tryptophan metabolites 3-hydroxyanthranilate, cinnabarinate, quinolinate and picolinate. *Biochem Pharmacol* 1987 Jan 15; 36(2): 211-217.

Foster AC, Miller LP, Oldendorf WH and Schwarcz R. Studies on the disposition of quinolinic acid after intracerebral or systemic administration in the rat. *Exp. Neurol* 1984; 84: 428-440.

French JH, O'Brien D, Grueter B. Pyridoxine dependency in the syndrome of infantile myoclonic seizures. *Program of the 14th annual meeting of the American Academy of Neurology* 1962:40.

French JH, Grueter BB, Druckman R, O'Brien D. Pyridoxine and infantile myoclonic seizures. *Neurology* 1965; 15,2:101-112.

Fukui S, Schwarcz R, Rapoport SI, Takada Y, Smith QR. Blood-brain barrier transport of kynurenines: implication for brain synthesis and metabolism *J. Neurochem* 1991; 56: 2007-17.

Gal EM, Sherman AD. L-kynurenine: its synthesis and possible regulatory function in brain. *Neurochem Res* 1980; 5: 223-239.

Geigy Scientific Tables. C.Lentner, ed. CIBA-GEIGY Limited, Basle, Switzerland; 1984.

Guilarte TR and Wagner HN. Increased concentration of 3-hydrokynurenine in vitamin B_6 deficient neinatal rat brain. J.*Neurochem* 1987; 49: 1918-1926.

Hadberg B, Hamfelt A, Hansson O. Epileptic children with disturbed tryptophan metabolism treated with vitamin B6. *Lancet* 1964; I:145.

Hadberg B, Hamfelt A, Hansson O. Tryptophan load tests and pyridoxal-5'-phosphate levels in epileptic children. I. Non-progressive brain damage and degenerative brain disorders. *Acta Paediat Scan.* 1966; 55:363.

Hellstrom B, Vasella F. Tryptophan metabolism in infantile spasm. *Acta Paediatrica* 1962; 51: 665-673.

Heyes MP, Saito K, Devinsky O, Nadi NS. Kynurenine pathway metabolites in cerebrospinal fluid and serum in complex partial seizures. *Epilepsia* 1994;35:251-57.

Hughes PAM, Bower BD, Raine DN, Syed N. Metabolism of tryptophan in childhood epilepsy. *Arch. Dis. Child.* 1966; 41:642.

Hunt AD, Stokes J Jr, McCrory W. Pyridoxine dependency: report of a case of intractable convulsions in an infant controlled by pyridoxine. *Pediatrics* 1954; 13:140.

Jain S, Padma MV, Puri A, Jyuti, Maheshwari MC. Occurrence of epilepsies in family members of Indian probands with different epileptic syndromes. *Epilepsia* 1997; 38(2):237-44.

Jhamandas K, Boegman RJ, Beninger RJ and Bialik M. Quinolinate-induced cortical cholinergic damage: modulation by tryptophan metabolites. *Brain Res.* 1990; 529: 185-191.

KOhler C, Okuno E, Schwarcz R. Quinolinic acid metabolism in the brain: biochemical and immunochemical analysis. In: Stone TW, ed. *Quinolinic acid and the kynurenines*. CRC Press, Boca Raton, Florida, 1989: 63-76.

Lapin IP, Prakhie IB and Kiseleva IP. Excitatory effects of kynurenine and its metabolites, amino acid and convulsants administered into brain ventricles. *J.Neural Transm* 1982; 54: 229-238.

Levy RH, Mattson RH, Meldrum BS. *Antiepileptic drugs.* 4th edition. New York: Raven Press, 1995.

Lott IT, Coulombe T, DiPaolo RV. Vitamin B6 dependent seizures; pathology and chemical findings in the brain. *Neurol.* 1978; 28:47-54.

Lumeng L, Li TK. Mammalian vitamin B_6 metabolism: regulatory role of protein-binding and the hydrolysis of pyridoxine 5'-Phosphate in storage and transport. In: G.P. Tryfiates, ed. *Vitamin B_6, Metabolism and Role in Growth.* Food & Nutrition Press, Inc., Westport, CT 06880 USA, 1980: 27-51.

Luthman J, Vanerman E, Fredriksson G, Fornstedt-Wallin B. Regulation of quinolinic acid in

the normal rat brain by kynurenine pathway precursors. In: Filippini GA et al., ed. *Recent advances in tryptophan research*. Plenum Press, New York, 1996:229-239.

May CD. Vitamin B6 in human nutrition: a critique and an object lesson. *Pediatrics* 1954; 14:269-75.

Miranda AF. Protection against quinolinic acid-mediated excitotoxicity in nigrostriatal dopaminergic neurons by endogenous kynurenic acid. *Neuroscience* 1997; 78 (4): 967-75.

Mori S, Nagano M. Electron microscopic cytochemistry of alkaline phosphatase in neurons of rats. *Arch. Histol. Jpn* 1985; 48(4):389-97.

Moroni F, Lombari G, Moneti G, Aldinio C. The exitotoxin quinolinic acid is present in the brain of several mammals and its cortical content increases during the aging process. *Neuroscience Letters* 1984; 47: 51-55.

Naritsin DB, Kuniaki S, Markey SP, Cai Y Chen and Heyes MP. Metabolism of L-Triptophan to Kynurenate and Quinolinate in the Central Nervous System: Effects of 6-Chlorotryptophan and 4-Chloro-3-Hydroxyanthranilate. J. *Neurochem.* 1995; Vol. 65. 5: 2217-2226.

Niedermeyer E, Lopes da Silva F. *Electroencephalography, basic principles, clinical applications and related fields.* 1982, Urban and Schwarzenberg, Baltimore, Munich.

NG TB, Tam PP. Changes of acid and alkaline phosphatase activities in the developing mouse brain. *Biol. Neopate*. 1986; 50(2):107-13.

Perkins MN, Stone TW. An iontophoretic investigation of the actions of convulsant kynurenines and their interaction with the endogenous excitant quinolinic acid. *Brain Res.* 1982; 247: 184-187.

Perkins MN, Stone TW. Quinolinic acid: regional variations in neuronal sensitivity. *Brain Res* 1983; 259: 172-76.

Reinhard JF Jr, Erickson JB, Flanagan EM. Quinolinic acid in neurological disease: opportunities for novel drug discovery. *Advances in Pharmacology. Academic Press* 1994; v.

30;

Rosenberg LE. Vitamin-dependent genetic disease. In: McKusic VA, ed. *Medical Genetics* 1995: 73-8.

Scriver CR, Hutchison JH. The vitamin B6 deficiency syndrome in human infancy: biochemical and clinical observations. *Pediatrics* 1963; 31:240-50.

Scriver CR. Vitamin B6 deficiency and dependency in man. Am J Child Dis 1967; 113:109-114.

Scriver CR. Vitamin B6 deficiency. In: Bergsma D, ed. *Birth Defects Compendium.* The National Foundation March of Dimes, New York: Alan R. Liss 1979.

Schwarcz R, Speciale C, Okuno E, French ED and Kohler. Quinolinic acid: a pathogen in seizure disorders? In: Schwarcz R et al, ed. *Kynurenine and serotonine pathways. Progress in Tryptophan Research* .Plenum Press, 1991; 4: 697-707.

Shideler Ch. Vitamin B6: An Overview. *Am. J. Med. Technol.* 1983; 49:17-22.

Stone TW. Excitatory amino acids and dementia. In: Nicholson CD, ed. *Therapeutic Approaches to the Treatment of Dementia.* London, UK: Academic Press, 1993.

Stone TW. Neuropharmacology of quinolinic and kynurenic acids. *Pharmacology Review* 1993; 45 (3) 310-379.

Stone TW. and Perkins MN. Quinolinic acid: A potent, endogenous excitant at amino acid receptors in CNS. *Eur. J. Pharmacol* 1981; 72: 411-412.

Tam PP, Kwong WH. A study on the pattern of alkaline phosphatase activity correlated with observation on silver-impregnated structures in the developing mouse brain. *J. Anat,* 1987; 150:169-80.

Vecsei L, Miller J, Macgarvey U, Beal M. Kynurenine and probenecid inhibit pentylenetetrazol- and NMDLA-induced seizures and increase kynurenic acid concentration in the brain. *Brain Res.* Bull. 1992; 28: 233-238.

Whyte MP. Alkaline phosphatase and the measurement of bone formation. In: Frame B., Potts JT Jr. ed., *Clinical disorders of bone and mineral metabolism*. Excertta Medica, Amsterdam 1983:120-124.

Whyte MP, McAlister WH, Patton LS, Maggil LH, Fallon MD, Lorentz WB, Herrog HG. Enzyme replacement therapy for infantile hypophosphatasia attempted by intravenous infusion of alkaline phosphatase-rich Paget plasma: results in three additional patients. *J. Pediatr.* 1984; 105:926-33.

Wuarin JP, Kim YI, Cepeda C, Tasker JG, Walsh JP, Peacock WJ, Buchwald NA and Durek FE. Synaptic transmission in human neocortex removed for treatment of intractable epilepsy in children. Ann. *Neurol* 1990; 28: 503-511.

Yamamoto H. Studies on CSF tryptophan metabolism in infantile spasms. *Pediatr Neurol* 1991; 7: 411-4.

Yamamoto H, Horiguchi K, Egawa B, Murakami H. Studies on CSF kynureninase pathway abnormalities in patients with infantile spasms. *Ann Rep Jpn Epi Res Found* 1995; 7 172-7.

Yoshioka T, Inomata K, Tanaka O. Cytochemical localization of alkaline phosphatase in the ependyma of the rat medulla oblongata. *Cell Tissue Res.* 1985; 241(2):415-20.

Young SN, Joseph MH, Gauthier S. Studies on kynurenine in human cerebrospinal fluid: lowered levels in epilepsy. *J Neural Transm* 1983; 58: 193-204.